Estradiol increases IP₃ by a nongenomic mechanism in the smooth muscle cells from the rat oviduct

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Abstract

Estradiol (E₂) accelerates egg transport by a nongenomic action, requiring activation of estrogen receptor (ER) and successive cAMP and IP₃ production in the rat oviduct. Furthermore, E₂ increases IP₃ production in primary cultures of oviductal smooth muscle cells. As smooth muscle cells are the mechanical effectors for the accelerated oocyte transport induced by E₂ in the oviduct, herein we determined the mechanism by which E₂ increases IP₃ in these cells. Inhibition of protein synthesis by Actinomycin D did not affect the E₂-induced IP₃ increase, although this was blocked by the ER antagonist ICI182780 and the inhibitor of phospholipase C (PLC) ET-18-OCH₃. Immunoelectron microscopy for ESR1 or ESR2 showed that these receptors were associated with the plasma membrane, indicating compatible localization with E₂ nongenomic actions in the smooth muscle cells. Furthermore, ESR1 but not ESR2 agonist mimicked the effect of E₂ on the IP₃ level. Finally, E₂ stimulated the activity of a protein associated with the contractile tone, calcium/calmodulin-dependent protein kinase II (CaMKII), in the smooth muscle cells. We conclude that E₂ increases IP₃ by a nongenomic action operated by ESR1 and that involves the activation of PLC in the smooth muscle cells of the rat oviduct. This E₂ effect is associated with CaMKII activation in the smooth muscle cells, suggesting that IP₃ and CaMKII are involved in the contractile activity necessary to accelerate oviductal egg transport.

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Introduction

The canonical pathway by which estradiol (E₂) affects its target cells comprises binding to estrogen receptors (ER) and modification of gene expression and protein synthesis (Nilsson *et al.* 2001). However, some E₂ effects cannot be blocked by inhibitors of transcription or translation, or are too rapid to be due to changes in gene expression. These features do not appear compatible with the classical genomic actions and are termed nongenomic (Lössel *et al.* 2003, Lössel & Wheling 2003). E₂ nongenomic actions often involve the activation of G protein- α inhibitory (G α_i), stimulation of intracellular signal transduction pathways, including the generation of second messengers such as cAMP and IP₃, and activation of protein kinase A (PKA) or phospholipase C (PLC) in the E₂-target cells (Nadal *et al.* 2001, Wyckoff *et al.* 2001, Acconcia *et al.* 2005, Hill *et al.* 2010).

In the rat, a single injection of E₂ on day 1 of the cycle or pregnancy shortens oviductal transport of eggs from the normal 72–96 h to <24 h (Ortíz *et al.* 1979). We have previously demonstrated that RNA and protein synthesis inhibitors did not block E₂-induced oviductal

egg transport acceleration in unmated rats indicating that E₂ accelerates oviductal egg transport by a nongenomic mechanism (Orihuela *et al.* 2001). This E₂ nongenomic pathway involves a previous conversion of E₂ to methoxyestradiols through the activation of catechol-O-methyltransferase (COMT) (Parada-Bustamante *et al.* 2007, 2010), ER and adenylyl cyclase (AC) (Orihuela *et al.* 2003), and sequential production of cAMP and IP₃ (Orihuela *et al.* 2003, 2006, 2013).

The rat oviduct is mainly composed of an intrinsic layer of smooth muscle fibre, and an innermost highly folded mucosa formed by epithelial and stromal cells, the endosalpinx (reviewed in Croxatto (2002)). Transport of oocytes along the oviduct depends on the interaction between the secretory activity of the epithelial cells and the contractile activity of the smooth muscle cells (Moore & Croxatto 1988a,b, Ríos *et al.* 2007). The regulation of muscular motility is influenced by E₂ and requires activity of adrenergic nerves (Helm *et al.* 1982), nitric oxide (Perez Martinez *et al.* 2000), endothelin (Parada-Bustamante *et al.* 2012), oxytocin (Jankovic *et al.* 2001) and prostaglandins (Wijayagunawardane *et al.* 2003). These factors activate intracellular

signalling mainly associated with Ca^{2+} , cAMP or IP_3 (Jankovic *et al.* 2001, Barrera *et al.* 2004, Mohan *et al.* 2012). In this context, we have recently shown that, in the epithelial cells of the rat oviduct, E_2 increased cAMP production between 3 and 6 h, although IP_3 levels were not affected. Moreover, E_2 increased cAMP in the oviductal epithelial cells by a nongenomic mechanism that requires coupling between ESR1 and $\text{G}\alpha_i$ and stimulation of AC (Oróstica *et al.* 2014). Previous research has also shown that E_2 increased IP_3 levels in primary cultures of smooth muscle cells from the rat oviduct (Oróstica *et al.* 2014).

As smooth muscle cells are the mechanical effectors for the accelerated oocyte transport induced by E_2 in the oviduct (Croxatto 2002), this work determined the mechanism by which E_2 increases IP_3 in primary cultures of rat oviductal smooth muscle cells. Thus, we examined the effect of E_2 on the IP_3 levels in the smooth muscle cells under conditions in which protein synthesis, ER, $\text{G}\alpha_i$ or PLC activity were blocked by selective inhibitors. The subcellular localization of ESR1 and ESR2 as well as the effect of selective agonists for ESR1 or ESR2 on the IP_3 level was evaluated in the smooth muscle cells. Furthermore, expression of $\text{G}\alpha_i$ in the oviductal smooth muscle cells was also determined. Finally, the effect of E_2 on the activity of the enzyme associated to muscle contraction calcium-calmodulin protein kinase II (CaMKII) was determined in the primary cultures of smooth muscle cells.

Materials and methods

Animals

Locally bred Sprague-Dawley rats weighing 200–260 g were used. Animals were kept under controlled temperature (21–24 °C), and lights were on from 0700 to 2100 h. Water and pelleted rat chows were supplied *ad libitum*. Female mature rats were used in the estrous stage. The phases of the estrous cycle were determined by daily vaginal smears (Turner 1961) and all females were used after showing 2 consecutive 4-day cycles. The Ethical Committees of the Universidad de Santiago de Chile and the National Fund of Science (CONICYT-FONDECYT 1110662) approved the protocols for the care and manipulation of the animals.

Culture of primary smooth muscle cells from rat oviducts

For each replicate, 12 oviducts from six rats were excised and placed in pre-warmed Hanks's solution (Sigma Chemical) at pH 7.4. The whole oviduct was cut into small (4–8 mm²) pieces in Hanks's solution and then the smooth muscle cells were mechanically removed from the rest of the tissue and treated with Collagenase, Type I (Invitrogen) for 1 h to further disaggregation of the cells. The cell suspension was centrifuged at 1200 *g* during 5 min, washed, and seeded into six-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) in

DMEM/High Modified medium with 4.0 mM L-glutamine and 4.5 g/l glucose free of Phenol Red (Cat. No. SH30284.02, HyClone, Thermo Scientific, Waltham, MA, USA) supplemented with 10% (v/v) foetal bovine serum (Cat. No. SH30396.03, HyClone), 1 mM sodium pyruvate and 100 UI/ml penicillin and 100 µg/ml streptomycin. Smooth muscle cells were incubated at 37 °C in an atmosphere of 5% (v/v) CO_2 for at least 7 days to reach 75–80% confluence and their purity verified by immunofluorescence staining for cytokeratin (marker of epithelium cells), vimentin (marker of fibroblasts) or α -actin (marker of smooth muscle cells) antibodies.

Treatments

Primary cultures of smooth muscle cells were changed to medium without serum for 15 h before each treatment. Then, they were treated with 10^{-9} M E_2 (Sigma) or 0.01% ethanol as vehicle. Other primary cultures of smooth muscle cells were also incubated with the protein synthesis inhibitor 1 µg/µl Actinomycin D (ActD, Goldberg *et al.* 1962, Sigma), the ER antagonist 25 µg/µl ICI 182780 (Gagliardi & Collins 1993, Tocris Bioscience, Bristol, UK), the PLC inhibitor 1 µM ET-18-OCH₃ (Powis *et al.* 1992, Calbiochem, La Jolla, CA, USA) or the $\text{G}\alpha_i$ protein inhibitor pertussis toxin 1 µg/ml PTX (Lee *et al.* 2002, Sigma) as appropriate to each experiment. Furthermore, the ESR1 agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (10^{-7} – 10^{-5} M PPT, Sigma) or ESR2 agonist Diarylpropionitrile (10^{-7} – 10^{-5} M DPN, Sigma) were added to the primary smooth muscle cells. The concentrations of PPT and DPN used in this work were based considering that both drugs have lower affinity for ESR1 and ESR2 than E_2 (Harris *et al.* 2002, Frasor *et al.* 2003, Harrington *et al.* 2003). DMSO at 0.01% was used as a vehicle for the inhibitors and agonists because it is more efficient than ethanol at dissolving nonpolar or semi-polar drugs.

Extraction and measurement of IP_3

Primary smooth muscle cell cultures were sonicated in 100 µl of ice-cold 1 M trichloroacetic acid (TCA) and an aliquot was taken to measure protein concentration by the Bradford assay using BSA dissolved in 1 M TCA as standard (Bio-Rad). The remaining homogenate was then centrifuged for 10 min at 1000 *g* at 4 °C. The pellet was discarded and the supernatant was incubated for 15 min at room temperature. TCA was removed from the supernatant with 0.5 ml of a solution 1,1,2-Trichloro-trifluoroethane (TCTFE, Sigma)-Trioctylamine (Sigma), 3:1 (v/v). Levels of IP_3 were determined using IP_3 [³H] radioreceptor assay Kit, Cat. No. NEK064 (NEN Life Science Products, Boston, MA, USA). This kit is based on competition between non-radioactive IP_3 and a fixed quantity of [³H]- IP_3 , for a limited number of calf cerebellum IP_3 receptor binding sites. This allows the construction of a standard curve and the measurement of IP_3 levels in unknown samples.

Western blot

Polyclonal antibodies that recognize the phosphorylated state of CaMKII on Thr286 (anti-phospho-CaMKII, Cell Signaling Technology, Beverly, MA, USA) or total CaMKII (anti-CaMKII,

Abcam, Cambridge, UK) were used to assess activation of CaMKII. Smooth muscle cells were processed by duplicate to determine the activity of the CaMKII protein. Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol) supplemented with a protease inhibitor cocktail (Roche Diagnostics). The lysate was centrifuged at 4 °C for 10 min at 10 000 *g* and the pellet was discarded. Protein concentrations in the supernatant were measured by the Bradford assay (Bio-Rad). After boiling for 5 min, proteins (20 µg) were separated on 10% SDS-PAGE slab gels in a Mini PROTEAN electrophoretic chamber (Bio-Rad). Proteins resolved in the gels were electroblotted onto nitrocellulose membranes (Bio-Rad). The membranes were blocked 3 h in TTBS (100 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween-20) that contained 5% nonfat dry milk and were incubated overnight with 0.4 µg/ml rabbit anti-phospho-CaMKII (Cell Signaling Technology). The immunoreactive band was visualized by incubation for 1 h with 0.04 µg/ml goat anti-rabbit IgG antibody (Chemicon International, Temecula, CA, USA) conjugated to HRP, followed by the Enhanced Western Lighting Chemiluminescence reaction (PerkinElmer Life Sciences, Boston, MA, USA). Blots were stripped in 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl, pH 6.7 at 60 °C for 30 min, and reprobed with 0.2 µg/ml rabbit anti-CaMKII antibody and developed in a similar manner to ensure even loading. All blots were then digitalized and the relative level of phospho-CaMKII was normalized against total CaMKII. Oviductal samples without anti-phospho-CaMKII or anti-CaMKII antibody were included as negative controls.

Immunoelectron microscopy

A post-embedding immunogold-labelling method that preserves cellular integrity and maintains ER immunogenicity was used (Kessels *et al.* 1998, Qualmann *et al.* 2000, Orihuela *et al.* 2009). Primary smooth muscle cell cultures were fixed in 4% freshly depolymerised paraformaldehyde, 0.5% glutaraldehyde in 1 M phosphate buffer pH 7.4 containing 0.1 M saccharose, 1% DMSO and 1% CaCl₂ for 2–4 h at room temperature. The fixed samples were washed three times with phosphate buffer, dehydrated in a graded ethanol series and infiltrated with LR Gold (Plano, München, Germany). Subsequently, the samples were transferred to gelatin capsules filled with 0.8% (w/v) benzoyl peroxide in LR Gold and kept for polymerization at a pressure of 500 mmHg. The blocks were cured for 1–2 days at room temperature before sectioning with a Sorvall-2000 ultramicrotome using a diamond knife. The sections (50–80 nm) were mounted on formvar-coated nickel grids and incubated on droplets of 0.1 M glycine in PBS pH 7.6, and subsequently blocked with 1% bovine fetal serum for 2 h at room temperature. The grids were then incubated for 2 h with 4.0 µg/ml rabbit anti-ESR1 (MC-20, Santa Cruz Biotechnology) or 4.0 µg/ml anti-ESR2 (clone 68-4, Chemicon International). After washing with PBS, the preparations were incubated for 1 h with 0.3 µg/ml goat anti-rabbit immunoglobulin conjugated to 10 nm gold particles (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Sections were washed and contrasted with Reynolds stain (Reynolds 1963). All sections were examined using a Phillips-TECNAI 12 BIOTWIN EM Microscope (FEI

Company, Hillsboro, OR, USA) at 80 kV. As negative control for ESR1 and ESR2, the primary antibody was replaced by preimmune serum. At least ten areas of 63 µm² from different smooth muscle cells from the primary cultures were photographed and the photomicrographs were digitalized in an iBook computer (Apple Computer, Cupertino, CA, USA), and gold particles present only in the cells were counted using the image analysis Software Adobe Photoshop 7.0 (Adobe Systems) by an observer blinded to the different groups. The results of the immunolabelling are presented as the quotient of the number of gold particles present divided by the area and cell number analysed in three different cultures (Orihuela *et al.* 2009).

Immunofluorescence

Smooth muscle cells were fixed in cold 4% paraformaldehyde in PBS pH 7.4–7.6 for 2 h, transferred to 10% (w/v) saccharose in PBS for 60 min at 4 °C and 30% (w/v) saccharose in PBS at 4 °C overnight. Then, they were blocked with 1% PBS-BSA for 120 min, and incubated with 0.8 µg/ml mouse anti-cytokeratin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 2.5 µg/ml mouse anti-vimentin (Santa Cruz Biotechnology), 0.4 µg/ml mouse anti-α-actin (Santa Cruz Biotechnology) or 5.0 µg/ml mouse anti-Gα_i (Santa Cruz Biotechnology) antibodies. After washing with PBS, the preparations were incubated for 2 h with 0.5 µg/ml Alexa fluor 588-conjugated goat anti-mouse IgG (Invitrogen). Sections were washed and counterstained with 1 µg/ml of Hoechst 33342 (Thermo Scientific, Rockford, IL, USA) washing again and then mounted in Fluoromount G. As negative controls, the primary antibody was replaced by preimmune serum. As positive control for Gα_i we used samples of whole oviducts from rats on day 1 of the estrous cycle (Oróstica *et al.* 2013, 2014). All sections were visualized with an Optiphot Epifluorescence Microscope (Olympus).

Real-time PCR

Total RNA from primary secretory cell cultures was isolated using Trizol Reagent (Invitrogen). One microgram of total RNA of each sample was treated with Dnase I Amplification grade (Invitrogen). The single-strand cDNA was synthesized by reverse transcription using the Superscript III Reverse Transcriptase First Strand System for RT-PCR (Invitrogen), according to the manufacturer's protocol. The Light Cycler instrument (Roche Diagnostics) was used to quantify the relative gene expression of the E₂-target genes *c-fos* (Nilsson *et al.* 2001) in the oviductal smooth muscle cells; *Gapdh* was chosen as the housekeeping gene for load control. The SYBR Green I double-strand DNA binding dye (Roche Diagnostics) was the reagent of choice for these assays. Primers for *c-fos* were 5'-CCG AGA TTG CCA ATC TAC TG 3' (sense) and 5'-AGA AGG AAC CAG ACA GGT CC 3' (antisense) and for *Gapdh* were 5'-ACC ACA GTC CAT GCC ATC AC 3' (sense) and 5'-TCC ACC ACC CTG TTG CTG TA 3' (anti-sense). All real-time PCR assays were performed in duplicate. The thermal cycling conditions included an initial activation step at 95 °C for 5 min, followed by 40 cycles of denaturizing and annealing-amplification (95 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s) and finally one cycle of melting (60° up to 95 °C). The expression of *c-fos* was determined using the equation:

$Y = 2^{-\Delta C_p}$ (16) where Y is the relative expression, C_p (crossing point) is the cycle in the amplification reaction in which fluorescence begins to be exponential above the background base line, $-\Delta C_p$ is the result of subtracting C_p value of *c-fos* from C_p value of *gapdh* for each sample. To simplify the presentation of the data, the relative expression values were multiplied by 10^3 (Livak and Schmittgen 2001).

Statistical analysis

Data for IP_3 and CaMKII assays from cultured oviductal cells were replicated five times for each treatment (for each culture experiment, oviductal cells were recovered from a pool of six different rats). Statistical analysis was performed using a GraphPad Prism 5.0 Software program. All data are presented as mean $\pm$ S.E.M. These data followed a non-normal distribution (Kolmogorov–Smirnov test) and significant differences between groups were determined through the use of variance analysis by Friedman's test with subsequent *post-hoc* Wilcoxon signed-rank test. Significance was accepted at $P < 0.05$. On the other, the quantitative analysis of the ESR1 or ESR2 distribution was subjected to Kruskal–Wallis test, followed by Mann–Whitney's U tests for pairwise comparisons when overall significance was detected. Significance was accepted at $P < 0.05$.

Results

E₂ increased IP₃ production by a nongenomic action in the smooth muscle cells from the rat oviduct

Primary cultures of smooth cells from rat oviducts, with a purity of 90–95% (Fig. 1), were divided into the following treatment groups: i) ethanol + DMSO, ii) E_2 + DMSO, iii) ethanol + ActD and iv) E_2 + ActD. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP_3 as described in the 'Materials and methods' section.

Figure 2 shows that in the vehicle group, IP_3 production ranged from 114.1 ± 30.3 to 123.2 ± 26.7 fmol/ μ g of protein while in the E_2 -treated group it was increased at 6 h (523.2 ± 66.7 fmol/ μ g of protein) but not at 1, 3 or 9 h. Administration of ActD alone or concomitant with E_2 did not affect the time-course of IP_3 production in the control or in the E_2 -treated group.

E₂ increased IP₃ production through ER and PLC activation in the oviductal smooth muscle cells

In each experiment, primary cultures of smooth muscle cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) E_2 + DMSO, iii) ethanol + inhibitor and iv) E_2 + inhibitor. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP_3 as described in the 'Materials and methods' section.

Figure 3 shows that in the control groups, the IP_3 level ranged from 151.6 ± 26.3 to 193.5 ± 37.3 fmol/ μ g of protein while in the E_2 -treated groups it ranged from 488.3 ± 59.7 to 599.4 ± 71.3 fmol/ μ g of protein. Administration of ICI 182780 or ET-18-OCH₃ alone did not affect the basal IP_3 production although blocked the E_2 -simulated IP_3 increase at 6 h.

ESR1 and ESR2 are localized in association with the plasma membrane of the oviductal smooth muscle cells

Primary cultures of smooth muscle cells from rat oviducts with no treatment were processed by immunoelectron microscopy using specific antibodies for ESR1 and ESR2.

Figure 4A shows that immunoreactivity for ESR1 and ESR2 was found associated to the plasma membrane, cytoplasm and nucleus in the oviductal smooth muscle cells. Furthermore, Fig. 4B shows that the quantitative analysis of the ESR1 or ESR2 distribution was a higher number of ESR1 and ESR2-reacting gold particles in the nucleus than in the plasma membrane or cytoplasm of the smooth muscle cells.

Activation of ESR1 but not ESR2 mimic the effect of E₂ on the IP₃ production in the oviductal smooth muscle cells

Primary cultures of smooth muscle cells from rat oviducts were treated with DMSO, PPT (10^{-7} – 10^{-5} M) or DPN (10^{-7} – 10^{-5} M) during 0, 1, 3, 6 or 9 h and processed to measure the concentration of IP_3 as described in the 'Materials and methods' section. Other smooth muscle cells cultures were also treated with

Figure 1 Expression of α -actin in primary cultures of smooth muscle cells from rat oviducts. Representative photomicrographs of primary cultures of smooth muscle cells from rat oviducts were processed by immunofluorescence microscopy to detect expression of cytokeratin, vimentin or α -actin. Note that α -actin (red) were only expressed in smooth muscle cells. Nuclei were stained with Hoechst 33342 (blue). Negative controls of the immunoreactivity were incubated with preimmune serum.

Figure 2 Effect of Actinomycin D (ActD) on the E₂-induced IP₃ increase in the oviductal smooth muscle cells. Primary cultures of smooth muscle cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) E₂ + DMSO, iii) ethanol + ActD and iv) E₂ + ActD. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP₃ as described in the 'Materials and methods' section. Note that ActD did not block the effect of E₂ on the IP₃ level. This experiment consisted of five replicates. **P* < 0.05 with respect to each treatment group.

DMSO or DPN 10⁻⁷ M and 6 h later processed by Real-Time PCR to determine the mRNA level of *c-fos*.

Figure 5 shows that in the control group, the IP₃ production ranged from 120.3 ± 44.1 to 148.5 ± 50.6 fmol/μg of protein while treatment with PPT increased IP₃ level in a dose-dependent manner at 6 h without any effect at 0, 1, 3 or 9 h. On the other hand, administration of DPN had no effect on the IP₃ level at any time or concentration studied (Fig. 5), although it increased the mRNA level of *c-fos* (control: 51.4 ± 11.3 vs DPN: 210.7 ± 36.2 relative expression, *n* = 5).

Gα₁ protein is not required for the IP₃ production increase induced by E₂ in the oviductal smooth muscle cells

Primary cultures of smooth cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) E₂ + DMSO, iii) ethanol + PTX and iv) E₂ + PTX. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP₃ as described in the 'Materials and methods' section.

Figure 6A shows that in the control group, IP₃ production ranged from 124.9 ± 38.1 to 133.5 ± 36.9 fmol/μg of protein while in the E₂-treated group it was increased at 6 h (601.4 ± 98.5 fmol/μg of protein) but not at 1, 3 or 9 h. Administration of PTX alone or concomitant with E₂ did not affect the time-course of IP₃ production in the control or in the E₂-treated group.

Gα₁ protein is not expressed in the smooth muscle cells from the rat oviduct

Primary cultures from smooth muscle cells from rat oviducts were processed by immunofluorescence in

order to assess the immunoreactivity of Gα₁ in these cells. In addition, we used sections of whole oviducts from rats on day 1 of the oestrous cycle to corroborate presence of Gα₁ in the oviductal tissues. This experiment was replicated five times.

Figure 6B shows that immunoreactivity of Gα₁ was not found either in the primary cultures of the oviductal smooth muscle cells or in the myosalpinx layer of the whole oviduct. However, Gα₁ was expressed in the endosalpinx of the rat oviduct as previously reported by Oróstica *et al.* (2014).

E₂ induced activation of CaMKII in the oviductal smooth muscle cells

Primary cultures from rat oviductal secretory cells were treated with ethanol or E₂ 10⁻⁹ M and 6.5 h later the level of phosphorylated CaMKII (p-CaMKII) was assessed by immunoblot. As E₂ increases the IP₃ level at 6 h after

Figure 3 Effect of ICI 182780 or ET-18-OCH₃ on the E₂-induced IP₃ increase in the oviductal smooth muscle cells. In each experiment, primary cultures of smooth muscle cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) E₂ + DMSO, iii) ethanol + inhibitor and iv) E₂ + inhibitor. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP₃ as described in the 'Materials and methods' section. Note that ICI 182780 and ET-18-OCH₃ blocked the effect of E₂ on the level of IP₃ at 6 h. This experiment consisted of five replicates, **P* < 0.05 with respect to each treatment group.

Figure 4 Subcellular localization of ESR1 and ESR2 in the primary cultures of smooth muscle cells from rat oviducts. (A) Representative photomicrographs of primary cultures of smooth muscle cells from rat oviducts processed by immunoelectron microscopy with gold labeled-antibodies for ESR1 or ESR2. Arrows show gold particle labeling localized to the plasma membrane (PM), cytoplasm (C) and nucleus (N). Negative controls of the immunoreactivity were incubated with preimmune serum. ICS=intercellular space. (B) Density of gold particles (X±SE) observed for ESR1 and ESR2 in the plasma membrane (PM), cytoplasm (C) and nucleus (N). Note that density of ESR1 and ESR2 in the PM were lower than in the C and N. $a \neq b \neq c$, $P < 0.05$. Replicates of this experiment consisted of 12 cells from three different cell cultures (four individuals cells/well plate).

treatment, we consider that 6.5 h is a reasonable time to evaluate activation of CaMKII downstream of the IP_3 increase.

Figure 7 shows that E_2 increased the level of p-CaMKII in comparison with the vehicle group.

Discussion

The contribution of the different cell phenotypes of the rat oviduct on the E_2 nongenomic pathway associated with the cAMP- IP_3 signalling and involved in the accelerated egg transport is recently being disclosed (reviewed in Orihuela *et al.* (2013)). Here we show that E_2 increased IP_3 levels in the oviductal smooth muscle cells by a nongenomic mechanism because suppression of mRNA and protein synthesis by ActD did not prevent the effect of E_2 on the IP_3 level. Moreover, the E_2

nongenomic pathway that increases IP_3 requires activation of ER and PLC since blockade of ER by ICI 182780 and PLC by ET-18-OCH₃ reverted the E_2 -induced IP_3 increase in the oviductal smooth muscle cells. Previous works have shown that some E_2 nongenomic pathways are associated with changes in the turnover of inositol lipids that generates IP_3 from the hydrolysis of phosphatidylinositol 4,5-bisphosphate in several cell systems (Kisielewska *et al.* 1996, 1997, Razandi *et al.* 1999, Ariazi *et al.* 2010). Our findings show for the first time that a nongenomic action of E_2 associated with PLC- IP_3 signalling is also present in the smooth muscle cells of the mammalian oviduct. The effect of E_2 on the IP_3 level occurred from 6 h and declined at 9 h, indicating a transient action on the PLC- IP_3 signalling in the smooth muscle cells. A rapid turnover of IP_3 , inactivation of PLC or down-regulation of ER could explain the lack

Figure 5 Effect of selective agonists for ESR1 (PPT) or ESR2 (DPN) on the IP_3 level in primary cultures of smooth muscle cells from rat oviducts. Primary cultures of smooth muscle cells from rat oviducts were treated with DMSO, 10^{-7} – 10^{-5} M PPT or DPN during 0, 1, 3, 6 or 9 h and processed to measure the concentration of IP_3 as described in the 'Materials and methods' section. Note that PPT increased the IP_3 level in a concentration-dependent manner at 6 h after treatment while DPN had no any effect. This experiment consisted of five replicates. * $P < 0.05$, ** $P < 0.02$ and *** $P < 0.01$ with respect to each treatment group.

Figure 6 Effect of PTX on E₂-induced IP₃ increase and expression of Gα_i in the oviductal smooth muscle cells. (A) Primary cultures of smooth muscle cells from rat oviducts were divided into the following treatment groups: i) ethanol + DMSO, ii) E₂ + DMSO, iii) ethanol + PTX and iv) E₂ + PTX. At 0, 1, 3, 6 or 9 h after treatment, cultured cells were processed to measure the concentration of IP₃ as described in the 'Materials and methods' section. Note that PTX did not block the effect of E₂ on the IP₃ level. This experiment consisted of five replicates. **P* < 0.05 with respect to each treatment group. (B) Representative photomicrographs obtained from primary cultures of oviductal smooth muscle cells or whole oviduct processed by immunofluorescence to detect expression of Gα_i protein. Note that Gα_i (red) is only expressed in the endosalpinx (mainly composed of epithelium cells) layer of the whole oviduct. Nuclei were stained with Hoechst 33342 (blue). Negative controls of the immunoreactivity were incubated with preimmune serum. L, lumen; E, endosalpinx; M, myosalpinx.

of effect E₂ on the IP₃ level at 9 h; however, this remains to be determined.

In contrast to other reports that showed a rapid increase of IP₃ by E₂ in rat vaginal epithelial cells and HEPG2 cells (Singh & Gupta 1997, Marino *et al.* 1998), we found that E₂ has a time of latency of 6 h to exert its effects on the IP₃ production in the smooth muscle cells from the rat oviduct. Differences in the expression of the ER isoforms or in the signalling pathways between the different cell phenotypes may explain the delayed response to E₂ in the smooth muscle cells. We postulate that the E₂ nongenomic action that increases IP₃ appears as a secondary response to intracellular changes localized upstream of PLC activation in the oviductal smooth muscle cells. According to this assumption, we have recently shown that E₂-induced IP₃ increase is preceded by a cAMP decrease in smooth muscle cells of the rat oviduct (Oróstica *et al.* 2014). Alternatively, E₂ may be first metabolized into 2-methoxyestradiol to increase IP₃ production in the oviductal smooth muscle cells. In this context, various biological effects of E₂ including regulation of egg transport in the rat oviduct or modulation of the antihypertensive and neuroprotective effects of E₂, requires previous conversion from E₂ to 2ME in its target organs (reviewed in Dubey & Jackson (2001) and Parada-Bustamante *et al.* (2015)).

Inhibition of the ER activity did not affect basal IP₃ production in the oviductal smooth muscle cells, indicating that other ER-independent signalling pathways are acting to state basal IP₃ level. In according with this idea, various signalling pathways such as Angiotensin-II, arachidonic acid, endothelin-1 and norepinephrine regulate production of IP₃ (reviewed in Bolton (2006)). On the other hand, ET-18-OCH₃ alone had no effect on the IP₃ production in the oviductal smooth muscle cells. Since 13 mammal PLC subtypes have

actually been reported (Rhee 2001), it is probable that basal IP₃ production depends on an ET-18-OCH₃-insensitive PLC. In this context, it has been found that ET-18-OCH₃ is more effective at inhibiting membrane-associated PLC-β1 than PLC-γ1 localized in the cytosol of human fibroblasts (Powis *et al.* 1992).

Physiological effects of E₂ are mainly influenced by the differential distribution of ESR1 and ESR2 in its target organs. Our results showing localization of ESR1 and ESR2 in the cell membrane, cytoplasm and nucleus

Figure 7 Effect of E₂ on the CaMKII activation in primary cultures of smooth muscle cells from rat oviducts. Primary cultures of smooth muscle cells from rat oviducts were treated with ethanol or E₂ and 6.5 h later CaMKII activation was determined by western blot and densitometry using anti-phospho-CaMKII or total CaMKII antibodies. Note that E₂ treatment increased the relative phosphorylation of CaMKII in the smooth muscle cells. This experiment consisted of five replicates. **P* < 0.05 with respect to the control group.

indicate compatible localization of both ER subtypes with genomic and nongenomic actions of E_2 in the smooth muscle cells of the rat oviduct. These findings corroborate previous studies on the presence of ER in nuclear and extranuclear regions in the rat oviductal epithelium cells (Orihuela *et al.* 2009) and reinforces the concept that signalling pathways associated with E_2 nongenomic and genomic actions operate in the mammalian oviduct (Parada-Bustamante *et al.* 2010, 2012, Orihuela *et al.* 2013). The fact that a lower proportion of ESR1 and ESR2 was found in the plasma membrane compared with those present in the cytoplasm and nucleus of the oviductal smooth muscle cells is consistent with the notion that only a small pool of ER is responsible for the E_2 nongenomic actions (reviewed in Watson & Gametchu (2003) and Wehling *et al.* (2006)).

The functional relevance of ER subtypes on the E_2 -induced IP_3 increase in the oviductal smooth muscle cells was investigated utilizing selective agonists for ESR1 (Harris *et al.* 2002) or ESR2 (Frasor *et al.* 2003). We found that activation of ESR1 by PPT mimicked the effect of E_2 on the IP_3 levels while activation of ESR2 by DPN had no effect. This suggests that the nongenomic pathway by which E_2 increases IP_3 in the oviductal smooth muscle cells operates through ESR1 activation. This is in keeping with previous works showing that the nongenomic actions of estrogenic compounds require activation of ESR1 (reviewed in Marino *et al.* (2006), Moenter & Chu (2012) and Watson *et al.* (2014)). On the other hand, the E_2 nongenomic pathways associated with activation of ESR2 in the smooth muscle cells of the rat oviduct needs to be disclosed.

It has been documented that the $G\alpha_i$ subclass is the G protein most often linked with the E_2 nongenomic actions coupled with a presumptive ESR1 localized in extranuclear sites (Wyckoff *et al.* 2001, Kumar *et al.* 2007, Lin *et al.* 2011, Watson *et al.* 2012). However, the ADP-ribosylating agent pertussis toxin did not block the effect of E_2 on the IP_3 production in the smooth muscle cells, suggesting that heterotrimeric $G_{i/o}$ -type proteins are not required for this E_2 nongenomic action. This is corroborated by the fact that the $G\alpha_i$ protein was not expressed either in the primary cultures of smooth muscle cells or in the myosalpinx layer (mainly composed of smooth muscle fibers) of whole oviducts from estrous rats. $G\alpha_s$ -, Gq - or $G\beta\gamma$ -coupled receptors and tyrosine kinase receptors also activate PLC subtypes in a variety of cell systems (Zhu & Birnbaumer 1996, Yang *et al.* 2013). Furthermore, small Rho and Ras GTPases participate in the activation of the PLC- ϵ (Yang *et al.* 2013). Since differential expression of PLC subtypes and G proteins is unknown in the smooth muscle cells from the rat oviduct, further studies are necessary to establish the mechanism by which the E_2 -ER complex activate PLC in these cells.

Previous reports have shown that the cytokine Tumour Necrosis Factor- α (TNF- α) stimulated production and

release of contractile mediators as prostaglandins, endothelins and nitric oxide in the bovine oviduct (Wijayagunawardane & Miyamoto 2004, Szóstek *et al.* 2011). Moreover, TNF- α modulates the effect of E_2 on the oviductal egg transport and TNF- α receptors are present in the rat oviduct (Oróstica *et al.* 2013). It is probable that the E_2 nongenomic pathway that increases IP_3 could be associated with TNF- α signalling in the oviductal smooth muscle cells, but this needs to be further explored.

It is well known that binding of IP_3 to its specific receptors (types 1, 2 and 3) localized on the intracellular compartments (nucleus, plasma membrane or endoplasmic reticulum) participates in Ca^{2+} signalling by mediating intracellular Ca^{2+} release, which results in activation of proteins associated with smooth muscle contraction (Kamm & Stull 1985, Kim *et al.* 2000). In this context, CaMKII is a serine/threonine kinase that is activated by Ca^{2+} and Calmodulin and it is implicated in regulation of vascular tone through phosphorylation of contractile proteins, including myosin light chain kinase (MLCK), the 20-kD myosin light chain (LC20), phospholipase- α_2 and the α -subunit of Ca^{2+} channel (reviewed in Kim *et al.* (2008)). Furthermore, the synthetic estrogen, estradiol benzoate, is able to stimulate CaMKII activity in mouse hippocampus by a nongenomic mechanism (Sawai *et al.* 2002). Similarly, we found that E_2 increased CaMKII phosphorylation in the smooth muscle cells of the rat oviduct. To our knowledge, this is the first time that an effect of E_2 on the activity of CaMKII in the mammalian oviduct is reported. As E_2 activated CaMKII presumably downstream of the IP_3 increase, it is probable that this enzyme could be part of the PLC- IP_3 signalling cascades induced by estrogens to activate nongenomic actions in smooth muscle cells.

Several works have reported that E_2 exerts its vasoprotective actions regulating the contractile tone of the vascular, airway and myometrium smooth muscle cells (Kisielewska *et al.* 1996, Townsend *et al.* 2010, 2012, Cairrão *et al.* 2012, Holm *et al.* 2013). These effects are mainly associated with changes in the intracellular Ca^{2+} mobilization associated with cAMP and IP_3 signalling pathways (Kisielewska *et al.* 1996, 1997, Townsend *et al.* 2010, 2012). Since E_2 increases the frequency of myosalpinx contractions that accelerate oviductal egg transport in the rat (Moore & Croxatto 1988a,b), we can speculate that this E_2 effect involves IP_3 production and activation of CaMKII in the smooth muscle cells. This E_2 effect on the oviductal egg movement could be involved in the very early maternal-embryo interactions that occur in the uterine tissues necessary to the embryo implantation (Gomez & Muñoz 2015).

In summary, we have found that E_2 increases IP_3 by a nongenomic action operated by ESR1, and that involves activation of PLC in the smooth muscle cells of the rat oviduct. Furthermore, E_2 activates CaMKII presumably downstream of the IP_3 increase in the oviductal smooth muscle cells, suggesting that IP_3 and CaMKII are

involved in the contractile activity of the oviduct. These findings provide new evidence to understand the molecular mechanisms underlying the role of smooth muscle cells on the E₂ nongenomic action that accelerates egg transport in the rat oviduct.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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